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Comparison of ethyl glucuronide in hair with phosphatidylethanol in whole blood as post-mortem markers of alcohol abuse

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Abstract

Ethyl glucuronide (EtG) is a direct metabolite of ethanol and has been used as a marker of alcohol abuse in both urine and hair. This study investigated the value of EtG testing in post-mortem hair for diagnostic improvement of alcohol abuse in forensic medicine. Material from 70 consecutive medico-legal autopsies was collected in accordance with the recommendations on ethics by the Swedish National Board of Forensic Medicine. A method for determination of EtG in hair samples was developed using ultra performance liquid chromatography/electrospray tandem mass spectrometry (UPLC/ESI-MS/MS; LOQ, 2.5 pg/mg). The result of the EtG analysis was compared with the findings of phosphatidylethanol (PEth) in femoral whole blood, as measured by high performance liquid chromatography with an evaporative light-scattering detector (HPLC–ELSD; LOQ, 0.22 μmol/l). Evaluation of liver histology and anamnestic evidence of alcohol abuse of the deceased were taken in consideration for the interpretation. Measurable levels of EtG were present in 49 of the 70 autopsy cases whereas PEth was present in 36. Thirty-nine cases had EtG levels above the cutoff limit (≥30 pg/mg) compared with 29 for PEth (≥0.7 μmol/l). Fifteen cases had EtG as exclusive indicator for alcohol abuse compared with four cases for PEth. These findings suggest that measurements of EtG in hair may provide improved diagnostic information on alcohol abuse, due to a long retrospective time-window for detection and stability of EtG in hair in the decaying cadaver. However, an EtG level below the cutoff does not completely exclude previous alcohol abuse.

Keywords: Ethyl glucuronide; Hair; Phosphatidylethanol; Alcohol marker; Forensic medicine

1. Introduction

Alcohol is the most commonly used substance of abuse in Sweden [1], and approximately 3.5% of all deaths are estimated to be alcohol related [2]. Post-mortem investigations of alcohol drinking behavior are therefore performed as part of the interpretation of the circumstances and the cause of death in forensic medicine. The traditional indicators used when investigating alcohol abuse are liver histology and anamnestic evidence. However, the typical pathological findings of alcohol abuse that are widely assessed amongst forensic pathologists, such as fatty liver, hepatitis and cirrhosis, are not always present and tend to be non-specific [3]. Confirming anamnestic evidence of alcohol abuse obtained from police reports and medical records are therefore highly valuable for the investigation but may be absent. This indicates a need for a biochemical test in forensic medicine that can provide reliable evidence of previous alcohol abuse.

Phosphatidylethanol (PEth) is a group of phospholipids formed by action of phospholipase D (PLD) only in the presence of ethanol [4,5]. PEth is considered a promising long-term indicator of alcohol abuse, being detectable up to 14 days after sobriety [5]. In comparison to the traditional alcohol markers, such as carbohydrate-deficient transferrin (CDT), gamma-glutamyltransferase (GGT) and mean corpuscular volume (MCV), PEth has the advantage of being a derivative of ethanol which makes it significantly more alcohol specific [6–8]. The PEth concentration in post-mortem blood from

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medico-legal autopsies has previously been studied and was found to provide indication of alcohol abuse in cases where this would otherwise be difficult [9]. PEth has been used by the Swedish National Board of Forensic Medicine as a marker of alcohol abuse in medico-legal autopsies.

Ethyl glucuronide (EtG) is yet another alcohol marker which is directly related to the metabolism of ethanol. EtG is formed by conjugation of ethanol with activated glucuronic acid by UDP-glucuronosyl transferases [10], but constitutes only a very small fraction (<0.1%) of the ingested dose [11]. Recent studies yet support the use of EtG testing in hair as a marker of alcohol abuse [12–19]. In contrast to body fluids and tissues, hair is protected from the internal post-mortem changes and this, together with its known ability to retain foreign substances, makes hair a useful autopsy material for analysis. The EtG in hair may therefore be favorable in the investigation of alcohol abuse in forensic medicine.

The aim of the present study was to investigate the suitability of EtG measurement in post-mortem hair as a marker of previous alcohol abuse for diagnostic improvement in forensic medicine. This was achieved by comparing the findings of EtG in hair with that of PEth in femoral whole blood, as well as with traditional indicators of alcohol abuse such as liver histology and anamnestic evidence, in routine medico-legal autopsy cases. The method for EtG determination in hair samples was developed using ultra performance liquid chromatography/electrospray tandem mass spectrometry (UPLC/ESI-MS/MS).

2. Materials and methods

2.1. Autopsies and sampling

The study was performed in accordance with the recommendations on ethics by the Swedish National Board of Forensic Medicine and included material from 70 consecutive autopsies carried out at the Department of Forensic Medicine, Lund, between the 18th October and the 27th December 2006. In Sweden, the performance of medico-legal autopsies are decided by the police in all cases of fatal crimes and accidents, suicides, deceased individuals suspected for abuse of alcohol, drugs or narcotics, and in cases with cadavers showing putrefaction. Cadavers with sampling complications, severe putrefaction, a time of death longer than a fortnight, or with an age outside the range of 18–70 years were excluded from the present study. Blood was obtained by a femoral vein puncture and stored until analysis according to a method to inhibit post-mortem formation of PEth in the presence of ethanol [20]. Hair samples were collected from the posterior vertex, cut as close to the scalp as possible, folded in aluminum foil, and placed inside a paper envelope. The samples were then stored under dark and dry conditions at room temperature until processed further. Liver tissue samples were stored in formalin at room temperature until further treatment.

2.2. Reagents

For the EtG extraction and analysis, dichloromethane (analytical grade), methanol (gradient grade), acetonitrile (HPLC grade) and formic acid were used (Merck, Darmstadt, Germany). The internal standard (penta-deuterated EtG, EtG-D_5) and EtG were from Medichem (Steinenbronn, Germany) and were diluted in methanol. The deionised water was obtained from a Milli-Q Plus water purification system.

2.3. Instrumentation

The UPLC/ESI-MS/MS system used for the EtG analysis consisted of a Waters Acquity ultra performance liquid chromatograph connected to a Quattro Premier XE tandem mass spectrometer with an ESI source, and MassLynx™/TargetLynx™ Software version 4.1 (Waters, Milford, MA, USA). The liquid chromatography was performed with a 1.8 μm 2.1 mm × 100 mm high-strength silica (HSS) trifunctional C_{18} column (Waters Co), preceded by a 0.2-μm column filter (Waters Co). A binary gradient consisting of (A) 0.1% formic acid in water, pH 2.85 and (B) 100% acetonitrile was used at a flow rate of 400 μl/min. The gradient was run as the following scheme: 0 min, A 99%, B 1%; 2.0 min, A 88%, B 12%; 2.2 min, A 0%, B 100%; 2.21 min, A 98%, B 2%. The total run time for the method was 2.60 min. The conditions of the mass spectrometer were as follows: ESI source in the negative ion mode with nitrogen gas as nebulizer at temperature 400 °C; desolvation gas temperature (nitrogen), 400 °C; capillary voltage, 3.5 kV; multiplier voltage, 650 V; extractor voltage, −3 V; RF lens voltage, 1 V; cone gas flow (nitrogen), 50 l/h; desolvation gas flow, 1000 l/h; ion energy I, 0.5 V; ion energy II, 2.0 V; entrance and exit potential, 1 V; and collision gas (argon) flow, 0.35 ml/min. EtG identification was performed by monitoring the deprotonated molecule with the two most intense product ions, whereas EtG-D_5 was identified with the deprotenated molecule and only one precursor-to-product transition (Table 1). The *m/z* 221 → 84.9 and *m/z* 226 → 85 transitions were used as quantifiers for EtG and EtG-D_5, respectively.

2.4. Hair sample preparations

Hair samples (100 mg) of the proximal segment (≤5 cm) were washed and subjected to EtG extraction according to a method described elsewhere [19]. Accordingly, after organic solvent decontamination wash (dichloromethane and methanol) hair samples were cut into small fragments (1–2 mm) and incubated overnight with deionised water and internal standard followed by 2 h of ultrasonication. The supernatants containing EtG extract were then immediately transferred into vials and injected (injection volume, 5.5 μl) in the UPLC/ESI-MS/MS system. An 8-point calibration curve (5, 10, 20, 40, 100, 200, 1000 and 2000 pg/mg) for routine use was prepared by serial dilution of EtG stock solutions with deionised water added to a pool of EtG–negative hair. The EtG concentrations of the unknown samples were estimated from the peak area ratio between EtG and EtG-D_5 with the calibration curve as reference. Levels above 30 pg/mg were considered to indicate alcohol abuse as proposed in a previous study [16].

2.5. Validation

All validations were documented according to CLSI guidelines [21]. The linear correlation between the concentration and the area ratio to the internal standard for EtG was evaluated by analyzing an 11-point calibration curve covering 5–15,000 pg/mg prepared by serial dilution of EtG stock solutions with deionised water. The quantification imprecision for the EtG analysis was evaluated by intra- and inter-assay coefficient of variation (CV). Intra-day precision was determined by analyzing quality control (QC) samples of water solutions containing EtG (20 and 10,000 pg/mg) in three replicates, whereas values for the interday precision where obtained by analyzing the QC samples

Table 1
Mass spectrometric parameters for ethyl glucuronide (EtG) and penta-deuterated EtG (EtG-D_5)

Compound	Parent ion (*m/z*)	Product ion (*m/z*)	Cone (V)	Collision energy (eV)	Dwell time (s)
EtG	221.1	75.0	−20	14	0.100
	221.1	84.9	−20	16	0.100
EtG-D_5	226.1	85.0	−24	13	0.100